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*Exp Neurol*. Author manuscript; available in PMC 2012 February 24.

Published in final edited form as:
*Exp Neurol*. 2008 March ; 210(1): 274–279. doi:10.1016/j.expneurol.2007.10.021.

# Quiescent adult neural stem cells are exceptionally sensitive to cosmic radiation

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## Abstract

Generation of new neurons in the adult brain, a process that is likely to be essential for learning, memory, and mood regulation, is impaired by radiation. Therefore, radiation exposure might have not only such previously expected consequences as increased probability of developing cancer, but might also impair cognitive function and emotional stability. Radiation exposure is encountered in settings ranging from cancer therapy to space travel; evaluating the neurogenic risks of radiation requires identifying the at-risk populations of stem and progenitor cells in the adult brain. Here we have used a novel reporter mouse line to find that early neural progenitors are selectively affected by conditions simulating the space radiation environment. This is reflected both in a decrease in the number of these progenitors in the neurogenic regions and in an increase in the number of dying cells in these regions. Unexpectedly, we found that quiescent neural stem cells, rather than their rapidly dividing progeny, are most sensitive to radiation. Since these stem cells are responsible for adult neurogenesis, their death would have a profound impact on the production of new neurons in the irradiated adult brain. Our finding raises an important concern about cognitive and emotional risks associated with radiation exposure.

## INTRODUCTION

Dynamic changes in production of new neurons in the adult nervous system correlate with the action of drugs, hormones, age, stress, physical activity, enriched environment, and a plethora of other processes and stimuli (Abrous, et al., 2005, Kempermann, 2006, Lie, et al., 2004, Ming and Song, 2005). Moreover, recent evidence indicates that adult neurogenesis may be required for learning, memory, and regulation of mood (Drew and Hen, 2007,

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Santarelli, et al., 2003, Saxe, et al., 2006, Warner-Schmidt and Duman, 2006); thus, compromised neurogenesis may have a direct impact on cognitive and emotional functions.

Radiation can profoundly affect adult neurogenesis. It inhibits cell division and production of new neurons in the subventricular zone (SVZ) and the dentate gyrus (DG), the two main areas of persistent neurogenesis in the adult brain; moreover, suppressed levels of neurogenesis can be observed long after the exposure to radiation (Fike, et al., 2007, Marshall, et al., 2005, Mizumatsu, et al., 2003, Monje, et al., 2002, Rola, et al., 2004). Given the link between adult neurogenesis and cognitive functions, the radiation-induced suppression of neurogenesis may be causally related to the cognitive deficits observed after radiation therapy; this possibility is now being recognized and countermeasures are being developed to prevent these therapy-related side effects (Fike, et al., 2007, Monje, et al., 2003).

Similar considerations pertain to manned exploration of space. During spaceflights beyond low Earth orbit, astronauts are exposed to potentially carcinogenic and tissue damaging galactic cosmic rays, solar proton events, and secondary radiation that includes neutrons and recoil nuclei produced by nuclear reactions in spacecraft walls or in tissue (Cucinotta and Durante, 2006). Such radiation may present a significant health risk for human exploration of the moon and Mars. The carcinogenic and neurodegenerative risks of space radiation have been widely recognized; however, as with therapeutic radiation in the hospital setting, the association of impaired neurogenesis with diminished cognitive and emotional function in astronauts has not been sufficiently appreciated.

We addressed the space radiation-related risks to neurogenesis by simulating the space radiation environment and determining the alterations in neural stem and progenitor cells in the adult brain, thus identifying the at-risk populations of these cells. Unexpectedly, our results demonstrate that in the hippocampus, the quiescent stem-like cells, rather than their rapidly dividing progeny, constitute the most vulnerable cell population. This finding underscores a previously unappreciated risk to neural stem cells and raises concerns about the risks facing astronauts on long duration space missions.

## MATERIALS AND METHODS

### Transgenic mice

For evaluating the effect of radiation on neural stem and progenitor cells we used a nestin-CFPnuc reporter mouse line (Encinas, et al., 2006). These transgene mice were generated by a pronuclear injection into the fertilized oocytes from C57BL/6xBalb/cBy hybrid mice of a construct encoding CFP with nuclear localization domain, whose expression was regulated by the promoter and the second intron of the nestin gene and polyadenylation sequences from simian virus 40. Transgenic mice were repeatedly mated with C57BL/6 mice for more than 7 generations. The use of this reporter line for examining changes in adult neurogenesis is described in detail elsewhere (Encinas, et al., 2006). Use of animals was reviewed and approved by the Cold Spring Harbor Laboratory, Brookhaven National Laboratory and Kennedy Space Center Animal Use and Care Committees.

### Irradiation and analysis

Two-month old nestin-CFPnuc mice were irradiated head alone under anesthesia (isofluorane) at the NASA Space Radiation Laboratory, Upton New York. Mice were exposed to 0 or 100 cGy of 1 GeV/n Fe ions (LET: 148 keVp/μm; these doses correspond to an average fluence of 1–3 hits per cell), injected with BrdU (150 mg/kg), and sacrificed 24 h later. Mice were administered an overdose of chloral hydrate, and the tissues were fixed by transcardial perfusion with 30 ml of PBS, pH 7.4 followed by 30 ml of 4% (w/v)

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paraformaldehyde in PBS. The brains were removed, cut longitudinally into two hemispheres and postfixed with the same fixative for 3 h at room temperature, then transferred to PBS, and kept at 4°C. Serial sagittal sections, 50-μm thick, were obtained using a Vibratome 1500 (Vibratome, St. Louis, MO). For the amino-cupric-silver and caspase-3 staining of cell degeneration, brains were treated overnight with 20% glycerol and 2% dimethylsulfoxide to prevent freeze-artifacts and embedded in a gelatin matrix using MultiBrain™ Technology (NeuroScience Associates, Knoxville, TN). After curing, the block was rapidly frozen by immersion in isopentane chilled to −70°C with crushed dry ice, mounted on a freezing stage of an AO 860 sliding microtome and sectioned. Sections were collected sequentially into a 4x5 array of containers filled with Antigen Preserve solution (50% PBS pH 7.0, 50% Ethylene glycol, 1% Polyvinyl Pyrrolidone) for immunohistochemistry. The containers to be used for amino-cupric-silver staining (de Olmos, et al., 1994) were filled with 4% formaldehyde in 1.4% sodium cacodylate buffer, pH 7.4. After mounting, the sections were air dried, counterstained with neutral red, dehydrated, cleared in xylene and coverslipped.

Immunostaining was carried out following a standard procedure. The sections were incubated with blocking and permeabilization solution (PBS containing 0.2 % Triton-100X and 3% BSA) for 1 h at room temperature and then incubated overnight at 4°C with the primary antibodies (diluted in the same solution). After thorough washing with PBS, sections were incubated with secondary antibodies in PBS for 1 h at room temperature in darkness. After washing with PBS, the sections were mounted on gelatine-coated slides with DakoCytomation Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA). Those sections that were designated for the analysis of BrdU incorporation, were treated, before the immunostaining procedure, with 2M HCl for 30 min at 55°C, rinsed with PBS, treated with 1mM sodium tetraborate for 10 min at room temperature, and washed with PBS. For diaminobenzidine staining, the secondary antibodies were incubated in PBS for 1 h at room temperature. After washing, the sections were incubated with peroxidase-linked ABC (Vector Laboratories, Burlingame, CA.). The peroxidase activity was revealed using the Sigma Fast 3,3'-diaminobenzidine tetrahydrochloride with metal enhancer tablet set (Sigma-Aldrich, St Louis, MO). Following antibodies were used: chicken anti-GFP (Aves Laboratories, Tigard, OR) diluted 1:500; chicken anti-vimentin (Chemicon International, Temecula, CA) diluted 1:500; rat anti-BrdU (Serotec, Raleigh, NC) diluted 1:400; rabbit anti-caspase-3 (Cell Signaling, Beverly, MA) diluted 1:1000, rabbit anti-Iba1 (Wako, Richmond, VA) diluted 1:5000, AlexaFluor 488 goat anti-rat (Molecular Probes, Eugene, OR) diluted 1:500; Biotin-conjugated donkey anti-chicken (Vector Laboratories) diluted 1:200.

## Quantification

Quantitative analysis of cell populations was performed by means of design-based (assumption free, unbiased) stereology (Howell, et al., 2002, Peterson, 1999). Slices were collected using systematic-random sampling. One brain hemisphere was randomly selected per animal. The hemisphere was sliced sagittally, in a lateral-to-medial direction, from the beginning of the lateral ventricle to the midline, thus including the entire DG. The 50 μm slices were collected in 6 parallel sets, each set consisting of 12 slices, each slice 300 μm apart from the next. All cells of each type described ($BrdU^+$, QNPs, and ANPs) were counted in every slice under a 63x objective, excluding those in the uppermost focal plane. The number of cells from all the slices from one set were added up together, and then multiplied by 6 (the number of sets of slices per animal), thus representing the total number of cells per hemisphere; these numbers are given in the Results section. The volume of the space reference (the GCL + SGZ) was estimated using the Cavalieri-point method and no significant changes were found between control and radiated animals. All the

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immunostaining images were collected using an epifluorescence/bright field microscope (Carl Zeiss, Thornwood, NY) equipped with a digital camera and the corresponding software. All images were imported into Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA) in the tiff format. Brightness, contrast, and background were adjusted using the “brightness and contrast” and “levels” controls from the “image/adjustment” set of options.

### Statistical analysis

Statistical analysis (Student’s t-test) and graph plotting was performed using Sigmaplot 8.0 (SPSS Inc., Chicago, IL). The bars show the mean and the standard error of the mean (s.e.m), and the black dots show the data for individual animals. Differences were considered to be significant when $p \leq 0.05$.

## RESULTS AND DISCUSSION

We have recently developed a novel reporter mouse line designed to help identify neural stem and progenitor cells in the adult nervous system and accurately quantify changes in selected classes of precursor cells induced by pro- or anti-neurogenic stimuli. In these mice, regulatory elements of the nestin gene, a marker of neural stem and progenitor cells, drive the expression of cyan fluorescent protein (CFP) fused to a nuclear localization signal from SV40 (nestin-CFPnuc mice; (Encinas, et al., 2006). Nestin-driven transgene expression visualizes several subclasses of neural progenitors in the adult SVZ and DG, whereas the nuclear representation of the transgene-expressing cells greatly facilitates enumeration of these subclasses. We use this reporter line to identify the precursor cell populations targeted by antidepressant fluoxetine (Encinas, et al., 2006), by deep brain stimulation (Encinas et al., submitted) and by electroconvulsive shock (Park et al., unpublished), thus demonstrating its utility for the studies of neurogenesis.

To simulate the space radiation environment, we exposed nestin-CFPnuc mice to 100 cGy doses of 1 GeV/n $^{56}$Fe ions. After irradiation animals were injected with thymidine analogue 8-Bromo-deoxyuridine (BrdU) to label dividing cells and selected cell populations in the brain were analyzed 24 hours later. We focused on neurogenesis in the hippocampus, since this region of the brain is involved in learning, memory, and emotions.

Exposure to radiation resulted in a dramatic decrease (to 55%) in the number of BrdU-positive cells in the DG (Fig. 1A), in line with other reports demonstrating the radiation-induced decrease in the number of dividing cells in the DG (Mizumatsu, et al., 2003, Monje, et al., 2002, Rola, et al., 2004). In all cases, BrdU-positive cells were positioned in the subgranular zone (SGZ) and only occasionally in the hilus (Fig. 1E,H), indicating that radiation did not induce aberrant ectopic neurogenesis in the hippocampus.

Radiation also decreased the number of nestin-CFPnuc-expressing neural precursor cells in the DG (Fig. 1B,F,G,I,J). In the nestin-CFPnuc reporter animals, expression of CFP marks two classes of precursors in the DG, the quiescent neuronal progenitors (QNPs) and the amplifying neuronal progenitors (ANPs); these classes can be distinguished by their morphology and by their expression of glial fibrillary acidic protein (GFAP) and vimentin (Encinas, et al., 2006). The number of QNPs was decreased by irradiation by 45%; the reduction in the number of ANP cells, which are derived from the QNP cells, was much less (25%; Fig. 1C,D). There were no additional morphological or anatomical changes in the population of progenitor cells that were apparent as a result of irradiation (Fig. 1E–J). Thus, $^{56}$Fe radiation drastically decreased the number of dividing cells and neural progenitor cells in the DG, having the greatest effect on the quiescent population of neural progenitors.

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The loss of neural progenitors was also reflected in the highly increased number of dying and dead cells in the brain. These changes were evident when revealed both by amino cupric silver (a postmortem cell marker, showing disintegrative debris) and caspase-3 staining (a pre-mortem marker of apoptosis). The increase in cell degeneration was observed selectively in the neurogenic areas of the brain, the DG (Fig. 2A–F), the RMS (Fig. 2G–I), and the SVZ (data not shown); there were only a few caspase-3- or amino cupric silver-positive cells seen outside of these neurogenic areas of the brain, e.g., in the cortex (not shown). Within the DG, the dying cells were detected in the SGZ, where QNP and ANP cells are located (Fig. 2A–F). At this point, we cannot determine the identity of dying cells; thus the evidence for the death of QNP cells is indirect and is based on a dramatic decrease in their number. This leaves open several alternative interpretations such as decrease in the levels of expression of markers used to identify QNPs (note, however, that we probed these cells using several markers, GFP, GFAP, and vimentin); changes in the QNP morphology (note, however, that morphology of the remaining QNPs cells does not change); or their rapid transformation into other cells types e.g., ANPs (note, however, that the number of ANPs is also decreased).

The increase in the number of dying cells was observed 6 hours after irradiation, but not after 24 hours or 3 weeks, indicating a rapid clearance of radiation-damaged cells from the neurogenic areas, consistent with the observed apoptosis. Supporting these findings, we observed activated Iba1-positive microglial cells and cells resembling infiltrating macrophages selectively located in the SGZ (Fig. 2D–F); they were also seen in the SVZ and the RMS (Fig. 2I) but not in the cortex of the irradiated animals. As with the number of dying cells, these changes, normally associated with inflammation and tissue degeneration, were observed 6 h, but not 24 h or 3 weeks, after irradiation. Thus, the observed reduction in neural progenitor cells in the neurogenic areas is reciprocated by an increase in the number of dying and dead cells in these regions.

Together, our results demonstrate that QNPs, a population of quiescent stem-like cell in the hippocampus, are selectively killed by radiation. The concomitant loss of ANPs was expected since they represent a rapidly dividing cell population that is thus susceptible to various types of radiation. However, the finding that QNP cells, despite their low division rate, are particularly vulnerable to radiation, was unexpected as the current view holds that proliferating cells are more sensitive to radiation than quiescent ones. This suggests that additional mechanisms, not directly related to cell replication, may be responsible for this selective loss.

Our finding that a quiescent population of stem cells is killed by radiation is surprising in the light of the emerging concept of cancer stem cells (Dalerba, et al., 2007, Ignatova, et al., 2002, Jordan, et al., 2006, Vescovi, et al., 2006). This concept holds that tumors may re-arise after chemo- or radiotherapy from a small population of transformed cells with stem cell properties; it is assumed that these cells escape the cytotoxic regimens directed against rapidly dividing cells by virtue of their quiescence and then re-initiate the tumor. Our results on the exceptional sensitivity of QNPs to irradiation suggest that quiescence alone may not provide protection from radiation-induced cell death. Thus, there may be some other additional mechanisms operating in cancer stem cells (e.g., elevated repair capabilities or ability to exclude chemotherapeutic drugs) that are crucial for their apparent resistance to cytotoxic therapy.

Importantly, if the loss of QNPs, the most primitive and normally non-self-renewable progenitor class in the hippocampus, is not compensated (e.g., by increasing the rate of asymmetric divisions of the remaining QNPs or restoring their number through symmetric divisions), the number of new neurons and later, of all granule neurons in the DG may decline Such a delayed effect on neurogenesis has been observed in animals subjected to

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irradiation with $^{12}C$ and $^{56}Fe$ ions (Rola, et al., 2005) (at this point we are limited in our access to the radiation beam and will be able to address this important issue in our system in the future). Given the increasing evidence pointing to the role of adult neurogenesis in memory and mood control (e.g., the association between reduced neurogenesis in the DG and impairments in hippocampus-dependent cognitive tasks or the deficient response to antidepressant in mice with suppressed hippocampal neurogenesis (Santarelli, et al., 2003, Saxe, et al., 2006), the risk to stem-like QNP cells represents an important factor to consider when planning manned space missions or considering radiation exposure for therapeutic purposes. Further investigations should address the issue of whether a reduced flux expressed over a longer period that manned missions may expect during space exploration would have the same effects as the acute exposure levels used in these experiments. Our model offers a ground-based radiation-exposure test system that will help to assess radiation risk and to develop countermeasures, such as shielding and radioprotective drugs.

## Acknowledgments

We thank Barbara Mish, Adele Billups, Laura Thompson and Beatrice Pyatt for their assistance with the experiments. We are grateful to Yuri Lazebnik and Julian Banerji for discussions and comments. J.M.E. is a fellow of the Ministerio de Educación y Ciencia of Spain. Support to G.E. was provided by the Ira Hazan Fund and The Seraph Foundation and to D.S. by NINDS grant NS37556 and NHLBI grant 70143. This study was supported by grants from the Florida Space Research Institute, NASA Radiation Health Program and the National Space Biomedical Research Institute.

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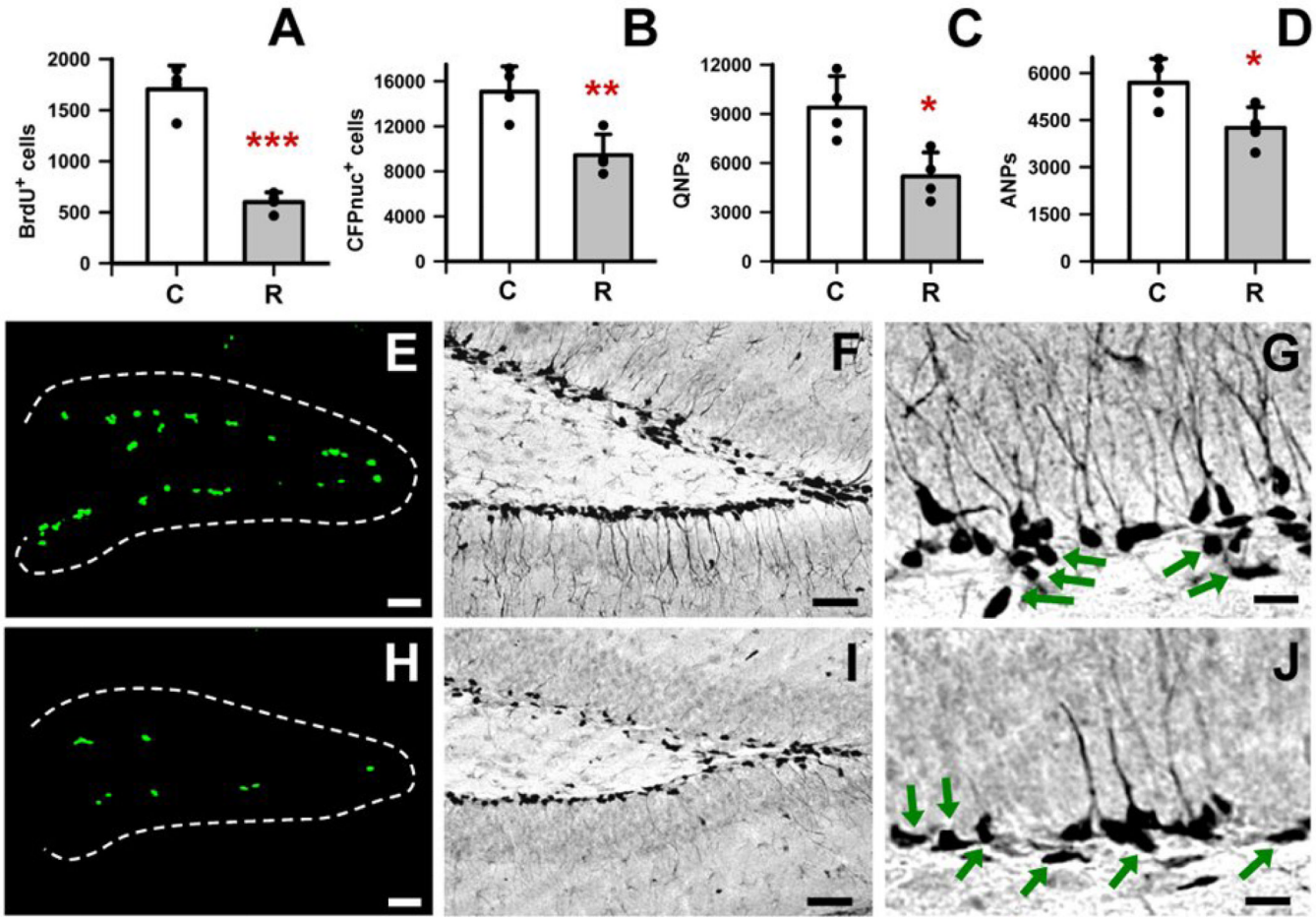


**Figure 1.**

**A–D.** $^{56}$Fe radiation decreases the number of BrdU-labeled cells (A), nestin-CFPnuc cells (B), QNP cells (C), and ANP cells (D) in the DG of nestin-CFPnuc mice. Mice were irradiated, injected with BrdU, and sacrificed 24 hours later. White bars correspond to the control group (C) and grey bars to the irradiated group (R). The results for individual animals are shown as black dots. Error bars show s.e.m. *p≤0.05, **p≤0.01, ***p≤0.0005.

**E–J.** Representative photomicrographs of DG sections from control (E, F, G) and irradiated (H, I, J) animals. (E and H) – sections stained for BrdU; dashed line outlines the external limits of the DG. (F, G, I, J) – QNP and ANP cells at lower (F, G) and higher (I, J) magnification. The soma and the nuclei (visualized by immunostaining against CFP) of both QNPs and ANPs are located in the subgranular zone. QNPs extend apical processes (immunostained with antibody to vimentin) which cross the granule cell layer and can thus be differentiated from the ANPs (arrows).

Scale bars are 20 μm in E, F, H I, and 5 μm in G, J.

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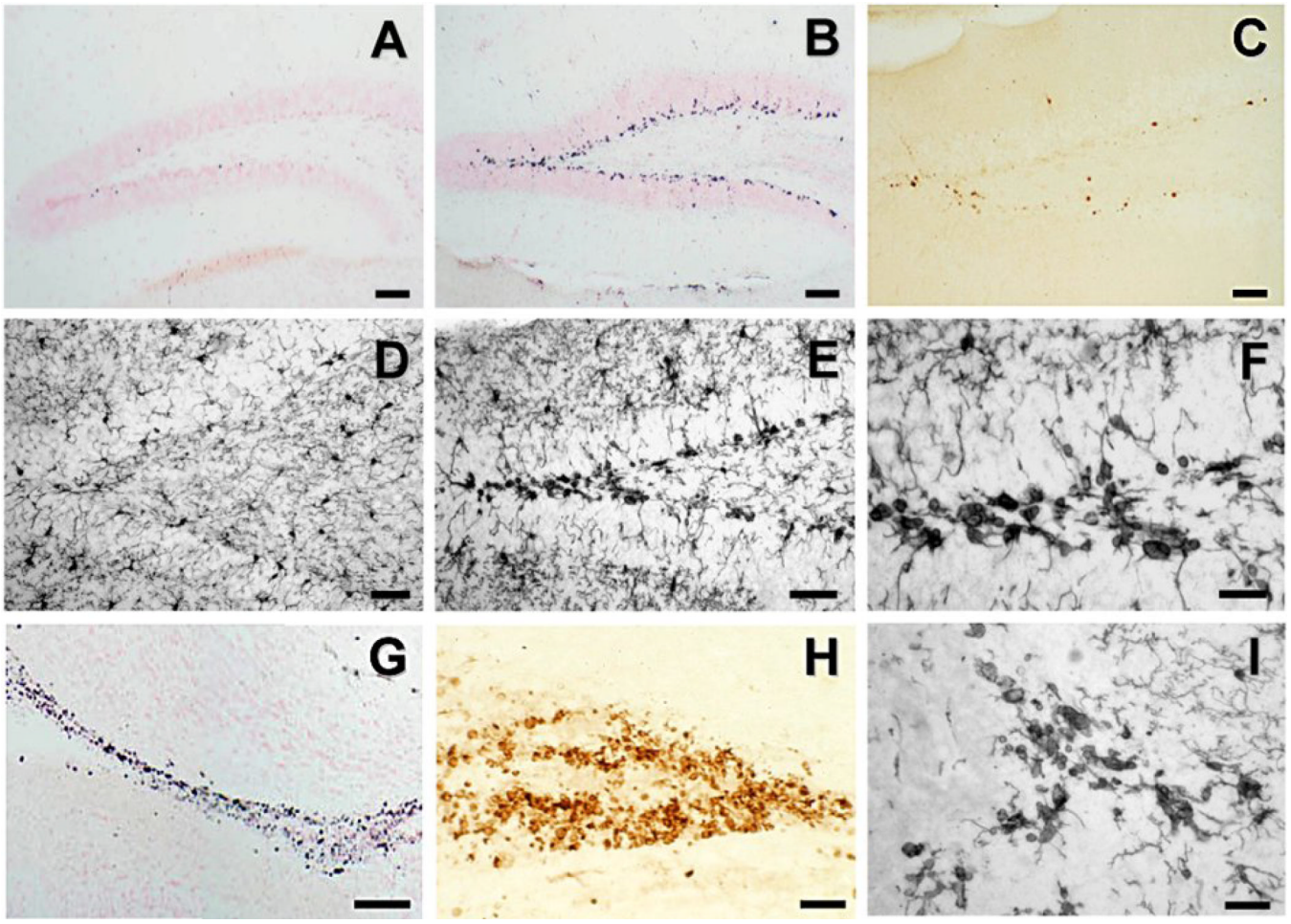


**Figure 2.**

**A–C.** Amino-cupric-silver stain for cell degeneration (A, B) and anti-caspase-3 staining for apoptotic cells (C) in the DG of control (A) and irradiated (B, C) mice, showing cell degeneration and apoptosis after irradiation.

**D–F.** Staining for microglial cells with anti-Iba1 antibody in the in the DG of control (D) and irradiated (E,F) mice; note altered morphology of microglial cells after irradiation.

**G–I.** Amino-cupric-silver (G), anti-caspase-3 (H), and anti Iba-1 (I) stainings in the RMS of radiated mice.

Scale bars are 50 μm in A–C; 40 μm in D, E, H; 30 μm in F, I; and 100 μm in G.